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Simultaneous determination of ginsenosides and lignans in Sheng-mai injection by ultra-performance liquid chromatography with diode array detection

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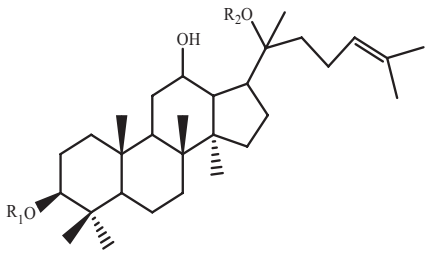
An ultra-performance liquid chromatography (UPLC) method with diode array detection was developed for simultaneous analysis of eight ginsenosides (ginsenosides Rg_1, Re, Rf, Rb_1, Rc, Rb_2, Rb_3, Rd) and one lignan (schizandrin) in Sheng-mai injection, a traditional Chinese medicine prescription widely used for the treatment of cardiovascular diseases. The chromatographic separation was performed on a Waters ACQUITY UPLC® HSS T3 column (1.8 μm, 100 mm × 2.1 mm i.d.) using a linear gradient elution over 28 min with a mixture of water and acetonitrile as the mobile phase. All calibration curves showed good linearity ($r^2 > 0.9998$) within the test ranges. Validation proved the repeatability of the method was good and recovery was satisfactory. The validated method was successfully applied to 12 batches of Sheng-mai injection. The results showed that there was a great variation among different samples. Principal component analysis (PCA) further proved considerable variations among the samples from different factories and suggested that schizandrin, ginsenosides Rb_1 and Rg_1 might have the greatest influence on the variation of 12 samples. In conclusion, these results demonstrated that the UPLC method proposed was very useful for the analysis and quality evaluation of Sheng-mai injection.

1. Introduction

Sheng-mai injection, which is derived from Sheng-mai San, is one of the most widely used TCM prescriptions in China for treating loss of energy, occurrence of excessive body fluids and coronary artery disease (Pharmacopoeia of PR China 2010) because of its excellent performance and low toxicity during long-term clinical use. It is made from three commonly used Chinese herbs, including *Radix et Rhizoma Ginseng, Radix Ophiopogonis* and *Fructus Schisandrae chinensis*. On the basis of previous research on the individual herbs, ginsenosides (Nadezhda et al. 2008; Nicola 2004) in *R. Ginseng,* ophiopogonin (Cheng et al. 2006; Yoshiaki et al. 1977) and ophiopogonone (Nguyen et al. 2003; Tran et al. 2010) in *R. Ophiopogonis* and lignans (Hee and Chul 2010; Lu and Chen 2009) in *F. Schisandrae chinensis* were considered to be the active components of Sheng-mai injection. However, the contents of ophiopogonin and ophiopogonone in Sheng-mai injection appeared to be very low on the basis of our experiments, which was consistent with results previously reported by Zheng et al. (2009). Tentatively, it might be concluded that the components of *R. Ophiopogonis* failed to be extracted during the preparation procedure of Sheng-mai injection.

Although the major components of Sheng-mai injection have been identified, no reliable analytical method has been available so far. A number of analytical methods have been reported for the quantification of ginsenosides and lignans, including TLC (Li et al. 1995; Yang et al. 2006), HPLC coupled with UV (Shi et al. 2007; Shi et al. 2010), ELSD (Man et al. 1996) and MS (He et al. 1997; Wang et al. 2007), however, most of these studies were aimed mainly at the analysis of crude drugs. In most cases, many types of constituents contained in the preparation, especially excipients, may interfere with the marker compounds in the assay, while the active components exist in only very small amounts. In addition, although some papers have reported effective quantification methods for related preparations, including Sheng-mai San (Chang et al. 2008) and Yi-Qi-Fu-Mai Preparation (Zhou et al. 2009), dosage forms were different in the proportion of prescription, extraction solvents and extraction methods for the crude drugs and the excipients, which led to discrepancies in internal compositions and pharmaceutical activities among the preparations. Therefore, it was necessary to develop a reliable method specially for Sheng-mai injection. Several analytical methods have been developed for determining the contents of ginsenosides and lignans in Sheng-mai injection, including spectrophotometry (Zhang et al. 1999) and HPLC-UV (Bai and Yu 2009; Xia et al. 2006; Zhang et al. 2009). However, previous reports focused mainly on single or a few marker constituents, insufficient to be evaluate for their overall effectiveness, because it is widely accepted that the therapeutic effect of TCM is usually based on multiple essential compo-



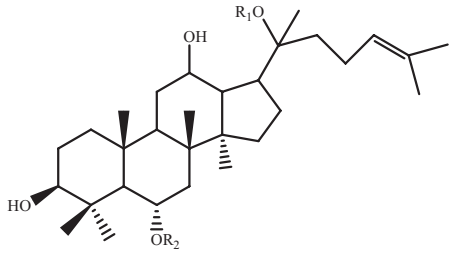
Ginsenoside Rb_1 R_1=-Glc^2-Glc, R_2=-Glc^6-Glc

Ginsenoside Rb_2 R_1=-Glc^2-Glc, R_2=-Glc^2-Arap

Ginsenoside Rb_3 R_1=-Glc^2-Glc, R_2=-Glc^2-Xyl

Ginsenoside Rc R_1=-Glc^2-Glc, R_2=-Glc^2-Araf

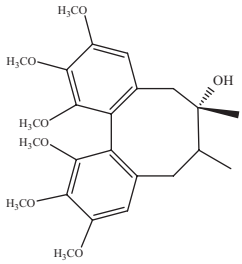
Ginsenoside Rd R_1=-Glc^2-Glc, R_2=-Glc



Ginsenoside Rg_1 R_1=-Glc, R_2=-Glc

Ginsenoside Re R_1=-Glc, R_2=-Glc^2-Rha

Ginsenoside Rf R_1=H, R_2=-Glc^2-Glc



Schizandrin

Fig. 1: Chemical structures of the nine investigated compounds

nents or their combination rather than any single component (Li et al. 2009). None of these methods was developed with a view to determining multiple active components simultaneously. Besides, these conventional methods suffered from low resolution, low sensitivity and long analysis times when they were used to analyze many ginsenosides that were similar in chemical structures. Considering the above problems, it was absolutely necessary to develop a satisfactory method for simultaneous detection of as many active compounds in Sheng-mai injection as possible to ensure its effectiveness and safety.

Recently, an improvement in chromatographic performance has been achieved by the introduction of ultra-performance liquid chromatography (UPLC), which shows great advantages over conventional high performance liquid chromatography (HPLC) due to very high-resolution separation and enhanced reproducibility over short periods of time, with little solvent consumption (Esmeralda and Alain 2009; Lucie et al. 2006). The growing interest in UPLC has been well demonstrated with around 600 papers published since 2003 (Davy et al. 2010).

Hence, the aim of our research work was to quantify multiple bioactive components simultaneously in Sheng-mai injection by UPLC. In this paper, we reported the first attempt to develop a rapid and reliable UPLC method to analyze nine bioactive components including ginsenosides Rg_1, Re, Rf, Rb_1, Rc, Rb_2, Rb_3, Rd and schizandrin in Sheng-mai injection. Their structures are shown in Fig. 1. Using this validated method, nine bioactive compounds were simultaneously determined within 28 min and the method was successfully applied to 12 batches of injections collected from different manufacturers in China. Further results of PCA were used to evaluate the difference among samples from various factories and various batches from the same factory. The established method can also be applied to the analysis of crude and other related botanical drugs.

2. Investigations, results and discussion

2.1. Optimization of UPLC conditions

To obtain chromatograms with better resolution of adjacent peaks within shorter time, the chromatographic conditions were optimized. A Waters ACQUITY UPLC® HSS T3 column could achieve better separation than the Waters ACQUITY UPLC® HSS C18 column. Because ginsenosides showed poor UV absorption and a low wavelength range (198–205 nm), acetonitrile was chosen as the organic phase and the detection wavelength was set at 203 nm. A series of mobile phases including acetonitrile–water in combination with acetic acid, phosphoric acid or ammonium acetate were examined. The results showed that acetonitrile–water was better than other systems because these mobile phase additive resulted in an unsteady baseline and the improvement on the resolution was insignificant. Additionally, different linear gradient profiles were applied to improve the separation of Sheng-mai injection by varying the solvent strength during the elution process and the optimum gradient was finally picked out through a large number of empirical attempts. In this experiment, differences in the separations were observed by changing the temperature of the column compartment over 20, 25, 30, 35, and 45 °C. The separation of ginsenoside Rg_1 and Re was better at lower temperature, while the resolution between ginsenoside Rb_1 and its adjacent peaks was improved at higher temperature. In such cases, it may be preferable to choose 30 °C in view of the overall resolution of Sheng-mai injection. Representative chromatograms of reference standard and a typical sample at the conditions described above are shown in Fig. 2. The chromatographic peaks were identified by comparing their retention time and UV spectra with those of each reference compound. In addition, spiking samples with reference compounds showed no addi-

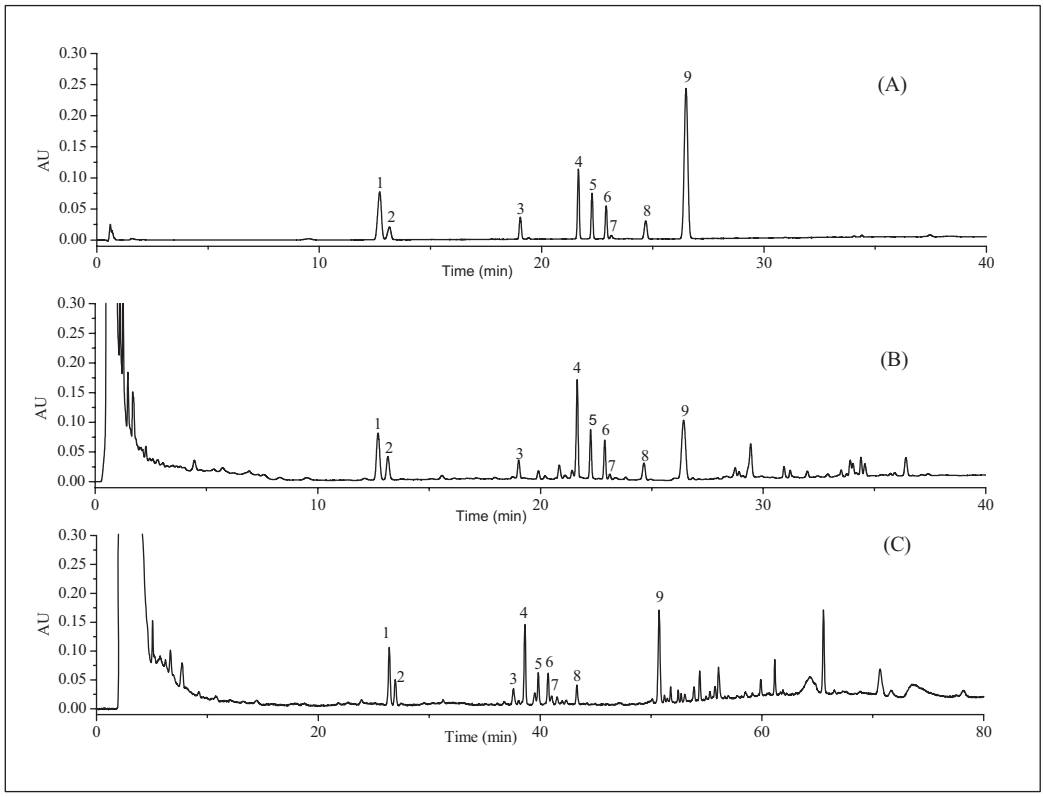


Fig. 2: Representative UPLC chromatograms of the standard mixture (A) and Sheng-mai injection (B); Representative HPLC chromatograms of Sheng-mai injection (C); 1 = ginsenoside Rg_1, 2 = Re, 3 = Rf, 4 = Rb_1, 5 = Rc, 6 = Rb_2, 7 = Rb_3, 8 = Rd, 9 = schizandrin

tional peaks, which further confirmed the identities of the analyte peaks.

2.2. Comparision of UPLC and HPLC

Due to the similar structures among the ginsenosides, reliable methods using HPLC for the separation of many ginsenosides were relatively time-consuming and inefficient, because they needed large amounts of organic solvents, sample and runtime. Comparing the chromatograms generated by UPLC and HPLC shown in Fig. 2, the established UPLC method needed only 28.0 min for analysis, which was half the analysis time of HPLC. The resolution of ginsenoside Rg_1 and Re was higher with UPLC than with HPLC. In addition, peaks 5, 6, 7 (ginsenoside Rc, Rb_1, Rb_2) and their adjacent peaks could not reach the baseline separation by HPLC and this would severely influence the accuracy of quantification, while these peaks could be seperated completely and determined accurately by UPLC. The combination of the shorter runtime and smaller flow rate (0.4 mL/min) reduced the solvent consumption to only 11.2 mL, whereas solvent usage for a single HPLC run was 55 mL. In our experiment, the signal of a 20 μL sample generated by HPLC was similar to that of a 5 μL sample by UPLC. Although many factors may influence sensitivity, such as the column, the detector, the mobile phase, the lamp time and so on, the results nevertheless indicated that UPLC can offer greater sensitivity than HPLC. With shorter runtime, lower solvent consumption, greater sensitivity and better resolution than HPLC, the UPLC method was effective for the comprehensive analysis of Sheng-mai injection.

2.3. Method validation

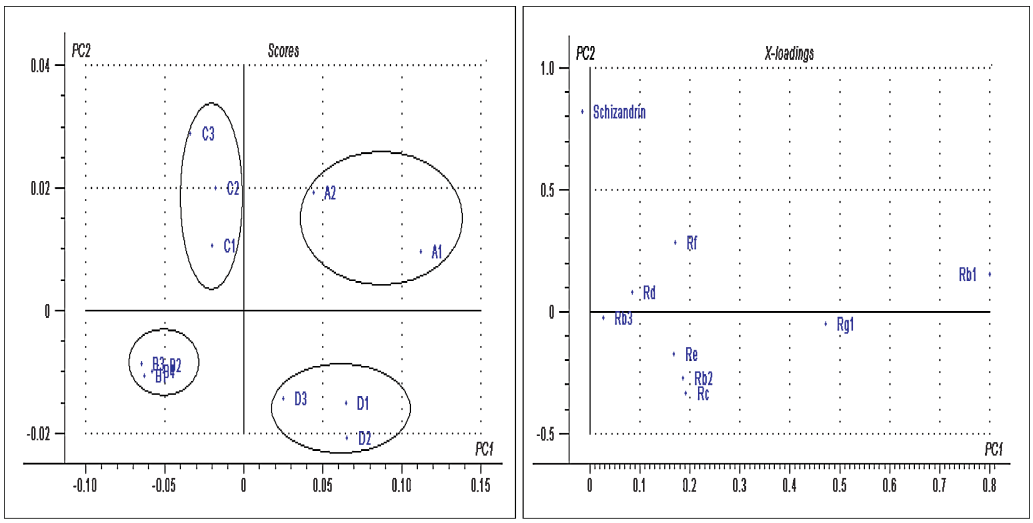
Specificity was assessed by comparing the UPLC chromatograms obtained from Sheng-mai injection, blank sample without *Fructus Schisandrae chinensis* and blank sample without *Radix et Rhizoma Ginseng*. There were no co-eluted peaks around the retention time of the nine analytes in the chromatograms, indicating that the selectivity of the method was acceptable for further quantification.

The linearity calibration curves for nine standards were assessed at six concentration levels and triplicate injections were applied at each concentration. Calibration curves were constructed by plotting the integrated chromatographic peak areas (Y) versus the corresponding concentration of the injected standard solution (X). Calibration plots indicated that all nine reference compounds showed good linearity ($r^2 > 0.9998$) over a relatively wide concentration range. The LODs (S/N ≥ 3) and LOQs (S/N ≥ 10) of the nine analytes ranged from 0.92 to 6.34 ng and from 2.88 to 20.6 ng, respectively. The results were summarized in Table 1.

The intra- and inter-day precisions were determined by analyzing calibration samples during a single day and on five consecutive days, respectively. To confirm the repeatability, six different solutions prepared from the same sample were analyzed. The recoveries were determined by the method of standard addition. Suitable amounts of the references were spiked into a sample of Sheng-mai injection, which had been accurately determined. The mixture was extracted and analyzed by the proposed procedure. The results of intra- and inter-day precisions, repeatability and accuracy were presented in Table 2. The results indicated that overall RSD values were less than 3%. Therefore, the established UPLC method was precise, accurate and sensitive for the simultaneous quantitative determination of nine compounds in Sheng-mai injection.

2.4. Determination of 12 samples and statistical analysis by PCA

Twelve batches of Sheng-mai injection from four pharmaceutical manufacturers were tested. As shown in Table 3, there



(A) (B)

Fig. 3: 2-D scores plot of two PCs from PCA on the 12 samples (No. 1–12 in Table 3) (A) and Corresponding loading plot of the nine analytes (B). PC1 and PC2 are the first two PCs from principal component analysis (PCA) using Unscrambler 9.8 software

were remarkable differences among the compositions of the nine bioactive compounds in different samples. The contents of eight ginsenosides varied with RSD 12.04%-24.90%, especially the content of schizandrin had a wide range of 0.002–0.043 mg/mL with a RSD 90.19%. This variation would be sure to result in the differences of internal quality and pharmaceutical actions.

In order to evaluate and discriminate 12 samples, the data were further analyzed by principal component analysis (PCA) using Unscrambler 9.8 software (CAMO software AS, Norway). As presented in Fig. 3A, five-components model explained 99.62% of the variance, with the first two components explaining 92.42% of the variability. The 2-D scores plot of the two principal components (Fig. 3A), where each coordinate represented a sample, showed the clear distribution of the 12 samples. From the scatter points, the 12 samples could be clustered into four groups, which were just classified according to different factories. The results showed that a great variation existed among the samples from different factories. The differences may arise from the different sources of the crude drugs, the detailed parameters of manufacturing technology and so on. More details will be investigated. However, the variation of components in different batches of the same factory was lower, especially company

Table 1: Linear regression, LODs and LOQs of nine bioactive compounds

Analytes	Linear regression data			LOQ	LOD
	Linearity range (μg/mL)	Regression equation	r^2	(ng)	(ng)
Rg_1	20.78−1039	$Y = 4.663 \times 10^3X - 2.923 \times 10^4$	0.9998	10.4	4.21
Re	6.128−306.4	$Y = 3.510 \times 10^3X + 3.686 \times 10^3$	0.9999	20.6	6.34
Rf	6.232−311.6	$Y = 3.642 \times 10^3X + 4.333 \times 10^3$	0.9999	6.23	2.31
Rb_1	19.14−957.0	$Y = 3.568 \times 10^3X - 2.045 \times 10^3$	0.9998	7.66	2.98
Rc	13.56−678.0	$Y = 3.221 \times 10^3X - 1.448 \times 10^4$	0.9998	6.78	2.85
Rb_2	10.52−526.0	$Y = 4.151 \times 10^3X - 1.448 \times 10^4$	0.9998	5.26	2.63
Rb_3	1.002−50.10	$Y = 3.721 \times 10^3X + 8.017 \times 10^2$	0.9999	6.18	2.04
Rd	6.136−306.8	$Y = 3.928 \times 10^3X + 3.720 \times 10^3$	0.9999	11.2	3.66
Schizandrin	4.368−218.4	$Y = 6.130 \times 10^4X + 3.713 \times 10^4$	0.9999	2.88	0.92

Table 2: Precision, repeatability and recovery of nine bioactive compounds

No.	Analytes	Precision		Repeatability	Recovery	
		Intra-day RSD (% $n = 6$)	Inter-day RSD (% $n = 5$)	RSD (%$n = 6$)	Mean	RSD (%)
1	Rg_1	0.16	0.51	0.92	99.68	1.51
2	Re	0.28	0.53	0.72	98.70	1.76
3	Rf	0.83	1.31	1.02	105.09	2.73
4	Rb_1	0.17	0.63	0.84	100.56	2.31
5	Rc	0.24	1.04	1.55	98.29	2.44
6	Rb_2	0.16	0.62	1.54	101.39	2.43
7	Rb_3	0.52	1.07	1.01	101.19	2.41
8	Rd	0.11	0.59	0.31	103.79	2.71
9	Schizandrin	0.13	0.55	1.61	100.67	1.75

C, which may attribute to the performance of good manufacture practice (GMP) in China in recent years. Furthermore, the loadings plot for PCA (Fig. 3B) indicated that schizandrin, ginsenosides Rb_1 and Rg_1 might have the greatest influence on the variation among the 12 batches samples. By controlling the contents of these three compounds, we could better control the internal quality of Sheng-mai injection, moreover, this would be helpful for us to find out the possible reason and carry out corresponding measures.

3. Experimental

3.1. Reagents and materials

HPLC grade acetonitrile and methanol were purchased from Merck Company (Merck, Darmstadt, Germany). Deionized water was prepared using a Milli-Q system (Millipore, MA, USA). Standards of ginsenoside Rg_1, Re, Rf, Rb_1, Rc, Rb_2, Rb_3, Rd and schizandrin were obtained from the National Institute for Food and Drug Control (Beijing, China). The purities of all compounds were above 98% and their structures are shown in Fig. 1. Twelve batches of Sheng-mai injection were collected from four pharmaceutical companies in China, named A, B, C and D, respectively. The intermediates for *Radix et Rhizoma Ginseng, Radix Ophiopogonis* and *Fructus Schisandrae chinensis* were supplied by manufacturer A and used for the preparation of blank samples without *Radix et Rhizoma Ginseng* and *Fructus Schisandrae chinensis*. The samples were deposited at the National Institute for Food and Drug Control (Beijing, China).

3.2. Preparation of mixed standard solution

Methanol stock solution of mixed standard was prepared, containing ginsenoside Rg_1 (1, 1.039 mg/mL), Re (2, 0.3064 mg/mL), Rf (3, 0.3116 mg/mL), Rb_1 (4, 0.957 mg/mL), Rc (5, 0.678 mg/mL), Rb_2 (6, 0.526 mg/mL), Rb_3 (7, 0.0501 mg/mL), Rd (8, 0.3068 mg/mL) and schizandrin (9, 0.2184 mg/mL). The flask was sealed by Parafilm (Parafilm, Chicago, IL, USA). The stock solution was further diluted to make working solutions. All the solutions were stored in the refrigerator at 4 °C before UPLC analysis.

3.3. Preparation of sample solution

Sheng-mai injections were injected directly for UPLC analysis after filtering through a 0.22 μm membrane. Blank samples (test samples without *Radix et Rhizoma Ginseng* and *Fructus Schisandrae chinensis*) were prepared respectively, by mixing the other two intermediate according to the formula.

3.4. UPLC and HPLC conditions

All analyses were performed on a Waters ACQUITY UPLC™ system (Waters Co., MA, USA) equipped with a binary solvent manager, sample manager, column compartment, photo diode array (PDA) detector and Waters Empower 2 software. The separation was performed on a Waters ACQUITY UPLC® HSS T3 column (1.8 μm, 100 mm × 2.1 mm i.d.). The detection wavelength was set at 203 nm. The mobile phase consisted of acetonitrile (A) and deionized water (B). The linear gradient was as follows: 0–10 min, 19% A; 10–25 min, 19–35% A; 25–35 min, 35–60% A, at a flow rate of 0.4 mL/min. After holding a solvent composition of 60% A for the next 5 min, the column was returned to its starting conditions. The column temperature was maintained at 30 °C and the injection volume was 5 μL.
The HPLC analysis was conducted on a Waters 2695–2996 system consisting of a quaternary pump, sample manager, column compartment and PDA detector. A Waters SymmetryShield™ RP18 (5 μm, 4.6 mm × 250 mm) was applied, using a linear gradient elution of acetonitrile (A) and water (B): 0–15 min, 19% A; 15–45 min, 19–40% A; 45–60 min, 40–60% A, at a flow rate of 1.0 mL/min. The column temperature was set at 30 °C, detection wavelength was at 203 nm and the injection volume was 20 μL.

3.5. Specificity

Specificity was assessed by comparing chromatograms obtained from the reference standard sample, injection sample and two blank samples without *Radix et Rhizoma Ginseng* and *Fructus Schisandrae chinensis*, respectively.

3.6. Calibration curves, LOD and LOQ

The stock solution containing nine components was diluted with methanol to six different concentrations within the ranges: 1) 20.78–1039 μg/mL; 2) 6.128–306.4 μg/mL; 3) 6.232–311.6 μg/mL; 4) 19.14–957.0 μg/mL; 5) 13.56–678.0 μg/mL; 6) 10.52–526.0 μg/mL; and 7) 1.002–50.10 μg/mL; 8) 6.136–306.8 μg/mL; 9) 4.368–218.4 μg/mL. Each concentration was analyzed in duplicate and then calibration curves were constructed by plotting peak areas of the components against their respective concentrations. The limit of detection (LOD) and limit of quantification (LOQ) were determined at a signal-to-noise ratio (S/N) of 3 and 10, respectively.

3.7. Precision, repeatability and accuracy

Intra- and inter-day variations were evaluated in order to determine the precision based on the relative standard deviation (RSD). For the intra-day variability test, the mixed standard solutions were analyzed for six replicates within a single day, while for the inter-day variability test, the solutions were examined in duplicates for consecutive 5 days.
To confirm repeatability, six solutions prepared from sample 1 were analyzed under the conditions described above. The RSD ($n = 6$) for repeatability was calculated.
The recovery test was performed by the standard addition method. Two mL of sample 1 and three different volumes of the stock solution of mixed standards at low (1.5 mL), middle (2.0 mL) and high (2.5 mL) levels were added into a 5 mL flask, then diluted to volume with water. The spiked samples were then shaken well, filtered and quantified according to the methods described above. Triplicate experiments were performed at each level.

3.8. Principal component analysis on the 12 samples

As a non-parametric and unsupervised pattern recognition method, PCA (Kong et al. 2010), reduces the dimensionality of the original data set by analyzing the correlation among a large number of variables in terms of a small number of underlying factors (principal components or PCs) without

Table 3: Contents (mg/mL) of nine bioactive compounds in twelve batches of Sheng-mai injection ($n = 3$)

Sample	Rg_1	Re	Rf	Rb_1	Rc	Rb_2	Rb_3	Rd	Schizandrin	Total saponins
A1	0.179	0.088	0.063	0.314	0.170	0.127	0.016	0.053	0.020	1.010
A2	0.157	0.082	0.059	0.257	0.141	0.108	0.013	0.043	0.024	0.860
B1	0.106	0.069	0.030	0.165	0.143	0.096	0.012	0.034	0.007	0.655
B2	0.108	0.069	0.036	0.171	0.147	0.101	0.012	0.036	0.009	0.680
B3	0.106	0.069	0.031	0.164	0.139	0.094	0.010	0.034	0.007	0.647
C1	0.132	0.078	0.047	0.196	0.151	0.095	0.012	0.040	0.027	0.751
C2	0.132	0.078	0.054	0.197	0.152	0.095	0.012	0.041	0.036	0.761
C3	0.127	0.074	0.056	0.184	0.147	0.087	0.011	0.039	0.043	0.725
C4	0.107	0.068	0.035	0.169	0.143	0.098	0.012	0.035	0.006	0.667
D1	0.175	0.099	0.058	0.258	0.172	0.119	0.015	0.044	0.003	0.940
D2	0.175	0.100	0.058	0.255	0.185	0.121	0.015	0.044	0.003	0.953
D3	0.157	0.089	0.051	0.228	0.164	0.106	0.013	0.039	0.002	0.847
Average	0.138	0.080	0.048	0.213	0.155	0.104	0.013	0.040	0.016	0.791
SD	0.029	0.012	0.012	0.049	0.015	0.013	0.002	0.005	0.014	0.127
RSD%	20.94	14.36	24.90	22.78	9.53	12.04	13.84	13.55	90.19	16.08

losing much information. PCA always results in score plots that provide a visual determination of the similarities and dissimilarities among the samples with respect to their biochemical composition. From the visualization of the data in a reduced dimensional space by this method, the samples can be separated and discriminated. The score plots of PC1 versus PC2 based on the contents of nine components in these 12 samples were examined for the separation or cluster relating to different batches of Sheng-mai injection. The numerical value of a loading of a given variable on a PC shows how much the variable has in common with that component. If the scores plot can discriminate the different groups of samples, the loadings plot can be used to express the components responsible for the separation among samples. The variables having the most influence on the scores plot are those furthest away from the main cluster of variables. In this study, loading plots of PCA were employed to find chemical components responsible for difference among different batches of Sheng-mai injection.

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